



EFFICIENT GENERATION OF ADENOVIRUS-BASED LIBRARIES BY POSITIVE SELECTION OF ADENOVIRAL RECOMBINANTS THROUGH ECTOPIC EXPRESSION OF THE ADENOVIRUS PROTEASE

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CROSS REFERENCE TO RELATED APPLICATION

This application is a Divisional of U.S. patent application Ser. No. 09/843,949 filed 04/30/2001, the entire content of which is incorporated by reference in this application.

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FIELD OF THE INVENTION

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The present invention relates to a method of generation of adenovirus recombinant vectors and adenovirus-based expression libraries, in particular to a method of generation of adenovirus recombinant vectors and adenovirus-based expression libraries by positive selection of adenovirus recombinant vectors through ectopic expression of the adenovirus protease.

BACKGROUND OF THE INVENTION

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The term "gene therapy" is usually understood to mean the process in which a gene is introduced into the somatic cells of an individual with the aim of being expressed in the cells, to produce some therapeutic effect. Initially this principle was applied to cases where an additional normal copy of a defective gene was provided to restore the synthesis of a missing protein, such as an enzyme. The concept of gene therapy has since been broadened to include several other approaches. In particular, the transferred gene (transgene) may code for a protein that is not necessarily missing but that may be of therapeutic benefit and difficult to administer exogenously, for example IL-2 or antitumor cytokines. This form of gene therapy aims to enhance *in vivo* production of potentially therapeutic proteins. This approach is similar to gene vaccination, where the transferred gene is introduced into the cells to express a protein acting as an antigen

inducing a protective immune response of the host's immune system. Another form of gene therapy involves transferring into cells non-physiological sequences which have antiviral activity, such as antisense oligonucleotides or sequences. Finally, so-called suicide genes can be transferred into undesirable cells (cancer cells or infected cells), to sensitize them to specific substances. When these substances are administered subsequently, they trigger selective destruction of the targeted cells.

Gene delivery systems which transfer the desired gene into the target cells are based either on physico-chemical or on biological methods. In each case the desired gene can be transferred into cells either *in vitro*, by extracting cells from an organ and reintroducing the cells transfected *in vitro* into the same organ or organism, or *in vivo*, i.e., directly into an appropriate tissue. Known physico-chemical methods of transfection include, for example, gene gun (biolistics), in situ naked DNA injections, complexes of DNA with DEAE-dextran or with nucleic proteins, liposomal DNA preparations, etc. Biological methods, considered to be a more reliable alternative to physico-chemical methods, rely on infectious agents as gene transfer vectors. In this group of methods, viruses have become infectious agents of choice, due to their inherent capability of infecting various cells. The transfer of a foreign gene by a viral vector is known as transduction of the gene.

Several virus classes have been tested for use as gene transfer vectors, including retroviruses (RSV, HMS, MMS, etc.), herpesviruses (e.g., HSV), poxviruses (vaccinia virus), adenoviruses (Ad, mainly derived from type 5 and 2 Ad) and adeno-associated viruses (AAV). Of those, adenovirus-based vectors are presently considered to be among the most promising viral vectors, due to their following properties, some of which are unique to this group of vectors: (i) adenovirus vectors do not require cell proliferation for expression of adenovirus proteins (i.e., are effective even in cells at the resting phase); (ii) adenovirus vectors do not integrate their DNA into the chromosomes of the cell, so their effect is impermanent and is unlikely to interfere with the cell's normal functions; (iii) adenovirus vectors can infect non-dividing or terminally differentiated cells, so they are applicable over a wide range of host cells; (iv) adenovirus vectors

show a transducing efficiency of almost 100% in a variety of animal cultured cells and in several organs of various species *in vivo*; (v) adenovirus vectors usually possess an ability to replicate to high titer, a feature important for the preparation of vector stocks suitable for the achievement of efficient transduction *in vivo*; (vi) adenovirus vectors can
5 accommodate large inserts of exogenous DNA (have a high cloning capacity); (vii) recombination events are rare for adenovirus vectors; (viii) there are no known associations of human malignancies or other serious health problems with adenovirus infections; (adenovirus type 5 is originally known to cause cold conditions in humans; live adenovirus of that type having the ability to replicate has been safely used as a
10 human vaccine (Top et al., *J.I.D.*, 124, 148-154; *J.I.D.*, 124, 155-160(1971)).

Structurally, adenoviruses are non-enveloped viruses, consisting of an external capsid and an internal core. Over 40 adenovirus subtypes have been isolated from humans and over 50 additional subtypes from other mammals and birds. All adenoviruses are
15 morphologically and structurally similar, even though they differ in some properties. Subtypes of human adenoviruses are designated according to serological response to infection. Of those, serotypes Ad2 and Ad5 have been studied most intensively, and used for gene transfer purposes since the 80s. Genetically, adenovirus is a double-stranded DNA virus with a linear genome of about 36 kb. The genome is classified into
20 early (E1 – E4) and late (L1 – L5) transcriptional regions (units). This classification is based on two temporal classes of viral proteins expressed during the early (E) and late (L) phases of virus replication, with viral DNA replication separating the two phases.

A viral gene transfer vector is a recombinant virus, usually a virus having a part of its
25 genome deleted and replaced with an expression cassette to be transferred into the host cell. Additionally to a foreign (exogenous) gene, the expression cassette comprises components necessary for a proper expression of the foreign gene. It contains at least a promoter sequence and a polyadenylation signal before and after the gene to be expressed. Other sequences necessary to regulate or enhance the gene expression
30 can be included in the cassette for specific applications.

The deletion of some parts of the viral genome may render the virus replication-incompetent, i.e., unable to multiply in the infected host cells. This highly desirable safety feature of viral vectors prevents the spread of the vector containing the recombinant material to the environment and protects the patient from an unintended viral infection and its pathological consequences. The replication-defective virus requires for its propagation either a complementing cell line (packaging cell line) or the presence of a helper virus, either of which serves to replace (restore *in trans*) the functions of the deleted part or parts of the viral genome. As it has been shown that the production of recombinant viral vectors free of replication-competent helper virus is difficult to achieve, the use of packaging cell lines for the propagation of replication-incompetent viral vectors is considered to be the best choice for gene therapy purposes.

Early adenovirus vectors (sometimes referred to as first generation adenovirus vectors, or singly deficient vectors) relied on deletions (and insertions) in coding regions E1 and/or E3 of the viral genome (see, for example, US 5,670,488; US 5,698,202; US 5,731,172). E3 deletion was usually performed to provide the necessary space for the insertion of foreign genes of a limited size. The E3 region is non-essential for virus growth in tissue culture, so that vectors deleted only in E3 region could be propagated in non-complementing cells. As E1 region is essential for the virus growth, E1-deleted vectors could only be propagated in complementing cells, such as human 293 cells (ATCC CRL 1573), a human embryonic kidney cell line containing the E1 region of human Ad5 DNA.

One of critical issues in the development of safe viral vectors is to prevent the generation of replication-competent virus during vector production in a packaging cell line or during the gene therapy treatment. This may happen as a result of a recombination event between the genome of the vector and that of the packaging cells, or of the vector and the wild-type virus present in the recipient cells of the patient or introduced as a contaminant in the process of producing the recombinant virus. On occasion, a recombination event could generate a replication-competent virus carrying the transgene, which virus might spread to the environment. Even though recombination

events are rare for E1-deleted adenovirus vectors, their *in vivo* replication and the ensuing risks could not be completely prevented, and generation of replication-competent adenovirus was demonstrated during the preparation of viral stocks. Another danger is the loss of replication deficiency (and the return to a phenotypic state of multiplication) through complementation *in trans* in some cells which produce proteins capable of replacing proteins encoded by the deleted regions of the viral genome. This was demonstrated for E1-deleted adenoviruses.

Attempts to improve the safety and cloning capacity of adenovirus vectors resulted in development of a new generation of multiply deficient adenovirus vectors (also referred to as second generation or multiply deleted vectors). Additionally to deletions in E1 and/or E3 coding regions, these vectors are also deleted in other regions of the viral genome essential for virus replication, such as early regions E2 and/or E4 (see, for example, WO 95/34671; US 5,700,470; WO 94/28152) or late regions L1 – L5 (see, for example, WO 95/02697). Other known approaches to improve the safety of adenovirus vectors include, for example, relocation of protein IX gene in E1-deleted adenovirus (US 5,707,618) and inactivation of the gene IVa2 in a multiply deleted adenovirus (WO 96/10088). All second generation adenovirus vectors are replication-deficient and require complementing cell lines for their propagation, to restore *in trans* the deleted or inactivated functions of the viral genome. More importantly, such vectors show an improved resistance to recombination when propagated in complementing cell lines or transferred into recipient cells of a patient, making recombination events virtually nonexistent and improving the safety of gene therapy treatments.

Since their development in the early '80s, adenovirus vectors (AdVs) have been widely used in gene transfer experiments for vaccination (reviewed in: Randrianarison-Jewtougoff et al., *Biologicals*, 23, 145 – 157 (1995)) and in gene therapy (reviewed in: Kovesdi et al., *Curr. Opin. in Biotech.*, 8, 583 – 589 (1997); Hitt et al., in: *The Development of Human Gene Therapy*, Cold Spring Harbor Laboratory Press, pp 60 – 86 (1999)). However, recent developments in the area of adenoviral vectors, such as the increase of insert size, the prolongation and the regulation of transgene expression,

as well as the modulation of AdV tropism have further expanded their applications. In particular, adenoviral vectors are now considered as one of the most powerful tools for functional genomics (reviewed in: Oualikene and Massie, in: Cell Engineering, vol. 2, Kluwer Publisher, pp 80 – 154 (2000); Wang et al., *Drug Discov. Today*, 5, 10 – 16 (2000)). Cloning and expressing numerous genes allows the generation of protein libraries useful for various applications, such as signal transduction studies or screening antisense DNA constructs. Such applications of AdVs require a cloning system in which generation and selection of recombinant mutants can be easily performed. An ideal method for the construction of AdV libraries would ensure that i) very large number of clones are generated following transfection of permissive cells, and ii) only recombinant viruses are selected. However, at present the construction of AdVs remains a cumbersome and lengthy process that is not readily amenable to the generation of large collection of clones.

Among the wide variety of methods used for the construction of recombinant AdV, several allow the generation of recombinant viruses without any background of parental genome (Ghosh-Choudhury et al., *Gene*, 50, 161 - 171 (1986); Bett et al., *Proc. Natl. Acad. Sci. USA*, 91, 8801 - 8806 (1994); Ketner et al., *Proc. Natl. Acad. Sci. USA*, 91, 6186 - 6190 (1994); Chartier et al., *Escherichia coli J. Virol.*, 70, 4805 - 4810 (1996); Crouzet et al., *Proc. Natl. Acad. Sci USA*, 94, 1414 – 1419, (1997); He et al., *Proc. Natl. Acad. Sci. USA*, 95, 2509 - 2514 (1998); Mizuguchi et al., *Hum. Gene Ther.*, 9, 2577 - 2583 (1998)). However, for all of these methods the number of viral clones generated is, at best, lower than 50 per μg of viral DNA. Only one method using the viral DNA-protein complex (DNA-TPC), which enhances the number of viral clones by up to 100-fold, was shown to provide large number of clones, albeit without selection for the recombinant ones (Miyake et al., *Proc. Natl. Acad. Sci. USA*, 93, 1320 - 1324 (1996)). This method relies on *in vivo* recombination in 293 cells of the viral genome co-transfected with a transfer vector harboring enough homologous sequences as well as cis-acting elements, such the left ITR which contains the origin of replication and the packaging region. Thus, even though it is currently possible to generate several thousand of viruses per μg of viral DNA, only a fraction of those will be recombinant.

To minimize the work involved in the screening process, reporter genes such as *E. coli* LacZ (Schaack et al., *J. Virol.*, 69, 3920 - 3923 (1995)) or the green/blue fluorescent proteins (GFP/BFP) from *A. victoria* (Massie et al., *Cytotechnology*, 28, 53 – 64 (1998)) can be used either in the viral genome as negative screen, or in the transfer vector as positive screen. Although useful, this approach still suffer from the intrinsic limitation that, in a library of several thousand of clones, an even larger number of parental viruses would have to be screened against, a process which is fairly time consuming. Furthermore, recombinant AdV are sometimes at growth disadvantage relative to the parental virus and these clones might be more difficult to isolate in a library, unless recombinant viruses are positively selected for growth.

Thus far, a positive selection system compatible with the generation of very large number of AdV clones has not yet been developed. One possible approach to do so would be to ectopically re-express an essential gene of adenovirus (which gene has been deleted at its native location) in such a way that only viral genomes that incorporated this gene would be able to grow in the selective environment (a positive selection). The present invention provides such a novel system for cloning DNA sequences in AdVs using the adenovirus protease as an example of the essential gene which can be used for the positive selection.

SUMMARY OF THE INVENTION

The present invention provides an adenovirus vector/packaging cell line system in which the vector replication is blocked by deletion of a single gene, not a viral transcriptional region, which deletion does not interfere with any other viral functions. The deleted gene is the gene of the adenovirus protease. The protease encoded by the deleted gene is expressed in a complementing (packaging) cell line through a regulatable expression cassette which induces no toxic effects in the cells, thus making the generation and production of the vector easier and efficient. As the deleted gene is highly specific of adenovirus, no complementation of the gene in transduced cells is to

be expected, which increases the safety and suitability of the protease gene deleted vectors for gene transfer purposes.

When additionally deleted for E1 region of adenoviral genome, the vectors of the invention are blocked for replication, but are capable of a single round of replication if deleted only for the protease gene. The latter feature permits an enhanced expression of the transgene in transduced cells, which may be of importance in some applications, for example to achieve localized enhanced expressions of transgenes (*in situ* tumor therapy) or efficient vaccinations without boosting.

In a preferred embodiment, the invention allows positive selection of E1-deleted, protease-deleted recombinant adenovirus vectors comprising an exogenous gene or an expressible piece of exogenous DNA, by providing an expression cassette comprising the protease gene and the exogenous gene or DNA under control of a suitable promoter, which may be a regulatable (e.g., inducible) promoter, inserted in place of E1 region in a shuttle vector. In another embodiment, the exogenous gene or expressible exogenous DNA is put into a separate expression cassette, under control of a suitable promoter. *In vivo* recombination of the shuttle vector with a protease-deleted adenoviral genome in suitable non-complementing cells generates viable recombinants only when rescuing the protease cloned in E1 region. Non-recombinant viral genomes are not able to grow due to the deletion of the protease gene, ensuring that only recombinant viral plaques are generated. This positive selection ensures generation of a large number of high purity recombinant adenovirus vectors and allows generation of adenovirus-based expression libraries with diversity exceeding 10^6 clones.

Consequently, it is an object of the present invention to provide novel cell lines capable of complementing *in trans* an adenovirus mutant deleted for the protease gene, which cell lines contain DNA expressing the adenovirus protease.

It is a further object of the present invention to provide a method for producing novel cell lines capable of complementing *in trans* an adenovirus mutant deleted for the protease gene, which cell lines contain DNA expressing the adenovirus protease.

5 It is a further object of the present invention to provide a method of using cell lines capable of complementing *in trans* an adenovirus mutant deleted for the protease gene and containing DNA expressing the adenovirus protease to generate and propagate adenovirus mutants deficient for the adenovirus protease gene.

10 It is a further object of the present invention to provide novel adenovirus mutants deleted for the adenovirus protease gene.

15 It is a further object of the present invention to provide novel adenovirus mutants deleted for the protease gene and at least one additional adenovirus gene or genomic region.

It is a further object of the present invention to provide novel adenovirus vectors for gene transfer, protein production, gene therapy and vaccination, said vectors deficient at least for the adenovirus protease gene and containing at least one exogenous gene
20 to be transferred to and expressed in a host cell.

It is a further object of the present invention to provide a novel method of generating recombinant adenovirus vectors comprising an exogenous gene or an expressible piece of exogenous DNA, by positive selection of recombinants deleted for the endogenous
25 protease, in which the protease gene is rescued by cloning the gene into another region of the adenoviral genome.

It is a further object of the present invention to provide a novel method of generating adenovirus-base expression libraries for expressing exogenous genes or expressible
30 pieces of exogenous DNA, by positive selection of recombinants deleted for the

endogenous protease, in which the protease gene is rescued by cloning the gene in another region of the adenoviral genome.

According to one aspect of the present invention, novel cell lines have been generated which are capable of expressing the Ad2 protease gene from a dicistronic expression cassette, under control of a tetracycline inducible promoter. The protease is expressed in these cells together with the green fluorescent protein (GFP), the latter used to facilitate cell cloning and expression monitoring. The novel cell lines have been prepared by transfecting derivatives of 293 cells with pieces of DNA encoding the Ad2 protease and GFP, selecting cells harboring these pieces (cells expressing the GFP) and amplifying them. The novel cell lines, stably expressing the Ad2 protease, produce amounts of protease equal to or greater than those reached after comparable infections by adenovirus. The biological activity of the novel cell lines has been demonstrated by their ability to fully support the reproduction of Ad2ts1 mutant, a temperature-sensitive mutant expressing a functionally defective protease and to restore normal yields of replication of two novel adenovirus mutants in which the protease gene has been deleted.

According to another aspect of the present invention, novel mutants of Ad5 deleted at least for the adenovirus protease gene have been generated. These novel mutants have been successfully propagated in the cell lines of the invention.

According to yet another aspect, the present invention provides a method of generating protease-deleted adenovirus mutants and adenovirus-based expression libraries having an exogenous gene or an expressible piece of exogenous DNA inserted in an early coding region, for example E1 coding region, using positive selection of recombinants obtained by *in vivo* recombination of adenoviral genome deleted for endogenous protease gene with a DNA construct capable of expressing the adenoviral protease and an exogenous gene or an expressible piece of exogenous DNA from an expression cassette or cassettes replacing the early coding region of the viral region or a part thereof.

According to still another aspect, the invention provides an adenoviral expression library comprising a plurality of recombinant adenoviruses, each recombinant adenovirus being deleted for an essential gene of a late transcriptional region of adenoviral genome, such as the protease gene, and having this essential gene expressibly cloned in a second transcriptional region of adenoviral genome, each recombinant adenovirus further comprising an expressible piece of exogenous DNA.

10 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a photograph showing expression of the protease by some clones of 293-tTA-PS and 293-rtTA-PS cells. Total protein extracts (30 µg) from different cell lines before (-) and after (+) induction were submitted to 14% SDS PAGE and transferred to nitrocellulose sheet. *E. coli* expressed Ad2 protease (lane 1: *E. coli*), adenovirus endogenous protease (lane 3: AdV) and non-transformed cells (lane 2: mock) were included as controls.

FIG. 1B is a photograph showing immunoblot of protein extracts of FIG. 1A. Proteins were revealed with an antiactin antibody to check that the same amount of protein was loaded per well.

FIG. 2 is a schematic representation of all molecular clonings performed to generate bacterial plasmids harboring protease deleted adenovirus regions. A PCR engineered protease deletion was introduced (after sequencing of the corresponding region) into pDE3 plasmid in which a 2378 bp upstream extension has been previously inserted by cloning of the RsrII/XhoI 6145 bp fragment from Ad5 genome.

FIG. 3 is a schematic representation of bacterial plasmids harboring protease deleted adenovirus regions and of the recombinations performed in *E. coli* to generate bacterial plasmids harboring protease deleted adenovirus genomes. The NdeI/XhoI fragment

from pDE3-ext-ΔPS plasmid was introduced by homologous recombination in *E. coli* with either pAdEasy1-βgal-GFP plasmid (which harbors an E1/E3 deleted Ad5 genome with reporter genes βgal of *E. coli* and GFP) or pTG3026 plasmid (which harbors an intact Ad5 genome) SgfI digested.

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FIG. 4 is a graph showing the effects of induction of protease expression on viability of cells of 293-PS cell lines. Six 6 cm Petri dishes were seeded with 2×10^5 cells of 293 and 293-PS tTA. Aliquots were examined for living/dead cells by trypan blue staining on day 0 (D0) through day 5 (D5). Results of overall cell growth of a typical experiment are plotted as the count of total living cells as a function of time (in days) for 293-tTA-PS cells, either induced (I) or not induced (NI), with 293-tTA as controls.

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FIG. 5 is a schematic representation of the molecular cloning performed to generate recombinant adenoviral vectors by positive selection with Ad protease. The recombinant represented here featured an E1-deletion. A shuttle vector, containing adenovirus 5 9.4 to 15.5 mu part of the genome to allow recombination, also harbored a triple expression cassette containing, among others, the protease gene and a foreign gene of interest (X) in place of the E1 region. After linearisation, the shuttle vector was cotransfected in a 293-derived cell line with a protease-deleted adenovirus genome cleaved in E1. Due to protease deletion, only genomes for which recombination, and thus the rescue of the protease gene, has occurred, produced viral plaques. The resulting recombinant viruses harbored no protease gene in L3 region, but the E1-cloned gene and the protease are ectopically expressed from the E1 region.

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FIG. 6 is a photograph showing a Coomassie blue stained gel demonstrating the ability of Ad5-ΔPS mutant to perform a single round of replication in non-complementing cells. A549 cells were inoculated at a MOI of 5 pfu with indicated mutants. 3 days later the cells were lysed in Laemmli buffer. 20 micrograms of protein extracts were loaded per well and migrated in a 12% acrylamide:bisacrylamide gel. Comparison of viral protein synthesized by the different mutants (i.e. hexon, 100K) shows that only Ad5-ΔPS

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mutant produces them in amounts similar to that of wild-type virus. This confirms the ability of this mutant to perform a single round of replication in non-complementing cells.

FIG. 7 is a graph showing the viral yields of different adenoviral mutants in A549 cells.

- 5 The ability of Ad5-ΔPS mutant to perform a single round of replication in non-complementing cells was further determined by titration of the same extracts as presented in Figure 6. A549 cells were inoculated at a MOI of 5 pfu with indicated mutants. 3 days later, cells were harvested and extracts were titrated in 293rtTA.PS.7. Titers are indicated in log values (0-9).

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Fig. 8 is a graph showing effects of the adenovirus protease expression on viral progeny yields. 293-rtTA cells were infected under three different induction conditions with Ad-TR5-PS-GFPq, AdTR6-PS-GFPq, and Ad5. Induction conditions are: uninduced (Dox⁻), induced with 1 μg/ml doxycycline at 5 hrs p.i. (Dox⁺ 5h) or 24 hrs p.i. (Dox⁺ 24h). The yields are shown as histograms and the error bar represents the standard mean calculated from 3 different points.

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Fig. 9 is a photograph of an immunoblot showing the level of ectopic expression of the protease (PS) gene by a recombinant AdV selected by ectopic expression of the PS gene. Total protein extracts (40 μg) from 293-rtTA cell lines infected with either AdTR5-PS-GFPq or AdTR6-PS-GFPq before (NI) (lanes 2 and 5) and after (I) induction for 5h (lanes 3 and 6) or 24h (lanes 4 and 7) after infection. All cells were harvested 48h after infection and the total proteins were analyzed by SDS-PAGE (14%) followed by immunoblotting. As controls, *E. coli* recombinant PS (lane 1), Ad endogenous PS (Ad5) (lane 8), parental 293-PS induced (lane 9) or non-induced (lane 10), and 293-rtTA cells (lane 11) were included. The upper panel (A) is an immunoblot revealed with rabbit polyclonal anti-PS serum whereas the lower panel (B) was revealed with an anti-actin monoclonal antibody to show equal loading.

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- 30 Fig. 10 is a graph comparing the effectiveness of infection/transfection and transfection/infection methods. To test the infection/transfection method, infections with

different MOIs of Ad5-ΔPS followed by transfection of pAdTR5-PS-GFPq (linearized by *Fse I*) at 5 hours post-infection were carried out. 293A cells cultivated in 60 mm dishes were infected at MOIs ranging from 10^{-2} TCID₅₀ (1 TCID₅₀ for 100 cells) to 10^{-7} . Five hours after infection, cells were washed, fresh medium was added, and cells were
5 transfected with 2μg of pAdTR5-PS-GFPq and 8 μg of carrier DNA using CaPO₄ precipitation method (Jordan et al., *Nucleic Acids Res.*, 24, 596 - 601 (1996)). Cells were washed after O/N incubation and fresh medium was added. To test the transfection/infection method, transfections with the same plasmid followed by infection
10 16 hours post-transfection were assessed. Cells were first transfected as described above and, after O/N incubation, cells were infected with same MOIs as above. Five days later, cells were freeze-thawed 3 times and titers of generated AdV were determined by plaque assay as detailed in Massie et al. (*Cytotechnology*, 28, 53 – 64 (1998)). Viral yields in total pfu/ 10^6 cells are plotted as a function of the MOI used for infection.

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Fig. 11 is a graph showing effects of MOIs and harvest times on viral yield. To study the correlation between the MOIs used for infection and the AdV yields on each day post-infection with the infection/transfection method, five 60 mm Petri dishes of 293A cells were infected at MOIs varying from 10^{-2} to 10^{-4} TCID₅₀, and then transfected with
20 linearized pAdTR5-PS-GFPq 5 hours later. The cells of each batch were split in 5 wells during the following days. On day 1, 2, 3, 4 and 6 post-infection, one well corresponding at each MOI was frozen. Titers of generated AdVs were determined by plaque assay. Viral yields in total pfu/ 10^6 cells are plotted as a function of the MOI and the day post-infection.

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Fig. 12 is a graph showing the diversity of recombinant library generated by the infection/transfection method. The diversity of libraries generated using the infection/transfection method was determined by establishing the minimal number of cells required to generate one recombinant. This was done using the 96 well plate
30 format. To optimize the yield of recombinant, the number of cells per well and the MOI were varied simultaneously. One hundred μl of decreasing log₂ dilutions of 293A cells,

starting at 10^4 cells/100 μ l were plated into 10 wells of 96 well plates. The next day, cells were infected with 50 μ l of Ad5- Δ PS, at MOIs 10^{-2} , 5×10^{-3} , 2.5×10^{-3} or 1.25×10^{-3} . Five hours later, DNA for transfection was prepared. Three μ g of pAdTR5-PS-GFPq *Fse* I digested and 12 μ g of carrier DNA were precipitated using the CaPO₄ method (Jordan et al., *supra*). The total volume was then brought to 2.5 ml with fresh medium 10 minutes later. Twenty-five μ l of this precipitate was dispensed into each well. The 96 well plates were incubated at 37°C for 5 days without any medium change. Cells were then harvested and subjected to 3 freeze-thaw cycles. For AdV detection from each well, fresh 293A cells (25000) were then plated and inoculated in the same plate format with 70 μ l of the products of each well. The plates were examined for up to 2 weeks for GFP+ cells as an indication of recombinant infection.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As used herein, the term "adenovirus" (Ad) means any adenovirus of human, mammalian, or avian origin (Mastadenovirus, Aviadenovirus families). Of those, human adenoviruses Ad2 and Ad5 are preferred, Ad5 being particularly preferred.

In the context of the present invention, the term "adenovirus protease" designates the protease of any adenovirus of human, mammalian, or avian origin, including analogues, homologues, mutants and isomers of such protease. The term "adenovirus protease gene" means the protease gene of any adenovirus of human, mammalian, or avian origin, including analogues, homologues, mutants and isomers of such gene. Even though minor differences exist between proteases of different adenoviruses, these proteases are interchangeable. Proteases of human adenoviruses Ad2 and Ad5 are preferred, the Ad2 protease being particularly preferred.

The adenovirus protease (PS) is one of the essential late viral genes involved in many steps of the virus cycle (reviewed in Webr J.M., in: The Molecular Repertoire of Adenoviruses, W. Doerfler and W. Boehm, eds., Springer Verlag, pp 227 – 235 (1995)).

First identified by studies on the Ad2ts1 temperature sensitive mutant (Weber J.M., *J. Virol*, 17, 462-471 (1976); Yeh-Kai et al., *J. Mol. Biol.*, 167, 217-222 (1983)), the adenovirus protease is a key enzyme in the adenovirus life cycle, serving for maturation of several proteins. Proteins cleaved by this enzyme are the pre-terminal protein (pTP), pVI, pVII, pVIII, pIIIa and the 11 K DNA binding proteins (Anderson et al., *J. Virol.*, 12, 241-252, (1973); Boudin et al., *Virology*, 101, 144-156 (1980); Tremblay et al., *Biochim. et Biophys. Acta*, 743, 239-245 (1983)). In addition to those substrates, the cleavage of viral 52K protein (Hasson et al., *J. Virol.*, 66, 6133-6142 (1992)) and of cellular cytokeratin 18 (Chen et al., *J. Virol.*, 67, 3507-3514 (1993)) play an important role in the viral cycle. Furthermore, the PS packaged within the mature virion is required for viral entry into host cells (Cotten et al., *Virology*, 213, 494-502, (1995); Greber et al., *EMBO J.*, 15, 1766-1777 (1996)) and for the release of virions from infected cells (Chen et al., *supra*). However, because the PS gene is not required for DNA replication, protease-deleted adenovirus mutants are capable of a single-round of replication in non-complementing host cells (which do not supply the protease *in trans*). As discussed more in detail further in the disclosure, this feature can be used to provide a positive selection method and system for recombinant AdVs deleted for the protease gene, by ectopic expression of the gene in other region of the adenoviral genome, in particular in E1 region.

Adenovirus protease deleted mutants provide numerous advantages for gene therapy and vaccination. Such mutants, whether deleted or not for additional genes (e.g., in the E1 coding region), are completely replication-deficient. Even though capable of cleaving some cellular proteins, the adenovirus protease is highly specific, making it extremely unlikely that the protease defect in the mutant could be overcome in a mammalian cell, an effect demonstrated for E1 deleted adenovirus mutants in some mammalian cells (HeLa and teratocarcinoma stem F9 cells: Imperiale et al., *Mol. Cell. Biol.*, 4, 867-874 (1984), Nevins et al., *Curr. Top. Microbiol. Immunol.*, 113, 15-19 (1984); embryonic carcinoma (EC): Keaveney et al., *Nature*, 365, 562-566 (1993)). This provides an increased safety level for therapeutic applications. For gene therapy applications, a complete blockage of replication of adenovirus can be reached by deleting the protease

gene together with other gene or genes essential to the virus growth, such as E1 coding region. Recombinant vectors deleted only for the protease and thus capable of a single round of replication provide interesting vectors for vaccination.

5 Adenoviruses deleted for the protease gene require for their propagation a cell line capable of providing the protease gene product *in trans*, such as the cell lines of the present invention. According to one preferred embodiment, 293S cell lines stably expressing the Ad2 protease (293-PS cells) have been generated. 293S cells were chosen for two reasons. Firstly, 293 cells allow the propagation of adenoviruses
10 simultaneously deleted in E1 and/or E3 coding region, such as recombinant adenovirus vectors for gene therapy applications. Secondly, the non-adherent phenotype of 293S cells is advantageous for a scale-up of preparation of deleted adenovirus (Gamier et al., *Cytotechnology*, 15, 145-155 (1994)), for example for the production of vector stocks. It would be apparent to those skilled in the art that other cell lines capable of hosting
15 adenoviruses, such as A549, 911, or BMAde1 (see US 5,891,690) would be also suitable for generating cell lines expressing the adenovirus protease gene.

According to the preferred embodiment of the invention, inducible promoters were chosen to achieve regulatable expression of the protease gene in the cell lines of the
20 invention, namely the tTA and rTA systems (Gossen et al., *Proc. Natl. Acad. Sci. USA*, 89, 5547-5551 (1992); Gossen et al., *Science*, 268, 1766-1769 (1995)). These systems allow for inducible expression of the gene, either by adding or withdrawing tetracycline to the cells. Regulatable expression cassettes were chosen because of the ability of the adenovirus protease to disrupt some components of the cellular cytoskeleton network
25 (Chen et al., *supra*). This function appears to play a key role in the phenotypic characteristics of adenovirus cytopathic effect, and thus may be at least deleterious for the host cells. A regulatable expression cassette makes it possible to limit the expression of the protease, at least at a high level, only to periods of time when the inducer is either added or withdrawn, so that the toxic effect of protease which could
30 hamper the generation or propagation of protease-deleted adenovirus is eliminated.

(Cells transfected by foreign plasmid DNA are stressed by transfection and much more sensitive to any toxic effects.)

5 For recombinant adenovirus vectors for gene therapy and vaccination, putting the transgene into a similar regulatable expression cassette provides a number of advantages. By choosing, for example, either tTA or rtTA regulation system, this allows the control of expression of the transgene either by administering tetracycline or by withdrawing its administration, respectively. This can be useful, for example, in vaccination of animals for which tetracycline is added on a regular basis to feeding.
10 Expression of the gene of interest can be in this case induced by withdrawing the administration of tetracycline during an appropriate period of time. It would be apparent to those skilled in the art that other regulatable promoters, such as ecdysone or corticosteroid responsive systems could be used for the practice of the invention.

15 The cell lines of the invention can be prepared by methods known to those skilled in the art, in particular by cotransfection of cells capable of hosting adenovirus with pieces of DNA encoding the adenovirus protease and pieces of DNA encoding a selection factor, incubating the cells, selecting cells expressing the selection factor, and amplifying those expressing the adenovirus protease. The selection factor can be anything which will
20 allow the selection of a cell, such as, for example, an antibiotic resistance protein.

According to the preferred embodiment, the novel complementing cell lines of the invention were produced by cotransfecting 293-tTA or 293-rtTA cells with plasmid pTR5/PS-DC/GFP (which contains a tetracycline regulatable (TR) promoter in a
25 dicistronic cassette (DC) with the GFP and the protease (PS) gene) and with plasmid pTKNeo (comprising the gene of resistance to geneticin (antibiotic G418)) or with plasmid p3'SS (comprising the gene of resistance to hygromycin), respectively, and selecting transfected cells with these antibiotics. Antibiotic-resistant colonies expressing the GFP protein were amplified and several of them selected for further analysis.

30

To facilitate the screening of recombinant clones, the adenovirus protease gene was expressed from a dicistronic cassette (Mosser et al., *Biotechniques*, 22, 150-161 (1997)) together with a reporter gene of *Aquorea victoria* green fluorescent protein (GFP) (Prasher et al., *Gene*, 111, 229-233 (1992); Heim et al., *Nature*, 373, 663-664 (1995)). After the first selection with an antibiotic (G418 or hygromycin), cells expressing GFP were selected for further studies by automated fluorescent cell sorting. This system allowed an efficient generation of 293 cell lines stably expressing the active Ad2 protease.

It will be apparent to those skilled in the art that the pieces of DNA encoding the adenovirus protease may be introduced into the cells using any DNA intracellular delivery system, such as, for example, recombinant plasmids, and by means of any transfection technique, such as calcium phosphate precipitation or liposome technology. Also, cells harboring pieces of DNA encoding the adenovirus protease may be made selectable using any suitable selection factor, such as the gene of resistance to an antibiotic, which gene can be transfected into the cells by a suitable recombinant plasmid.

To study the biological activity of the recombinant protein, complementation of the Ad2ts1 mutant (Weber, *J. Virol.*, 17, 462-471 (1976)) was examined. This mutant encodes a modified P137L protease which is active at the permissive temperature (33°C) and functionally defective at 39°C. Replication of the Ad2ts1 on 293-tTA-PS and 293-rtTA-PS cell lines allowed for restoration of yields similar to that of the wild-type virus. It was also shown that expression of the protease was not toxic to the cells but rather slightly impaired the normal cell growth. The novel cell lines were also shown to restore replication of two novel adenovirus mutants in which the protease gene has been deleted.

The novel adenovirus mutants deleted for the protease gene can be prepared by methods known to those skilled in the art. In general, the preparation of a virus mutant relies on preparing first the complete genome of the mutant by joining suitable pieces of

DNA, either by ligation in vitro or by recombination in a cell. In the latter case, several (usually two or three) fragments of adenoviral DNA containing regions of similarity (or overlap) are transfected into host cells, where they become recombined into a full-length viral genome. The fragments to be ligated or recombined may contain deletions and modifications with respect to the wild type viral genome, but must otherwise contain its entire length. The DNA of the recombinant virus so prepared is then transfected into suitable complementing cells capable of providing *in trans* viral functions missing from the transfected recombinant viral genome as a result of the deletions and modifications introduced into the wild type genome. The recombinant virus will multiply in these cells from which it can be subsequently released, for example by subjecting cells to several freeze-thaw cycles. Numerous variations of this general procedure are possible, as would be apparent to those skilled in the art.

According to the preferred embodiment, two novel Ad5 mutants (designated as Ad5CMVLacZ-CMVGFP- Δ PS and Ad5- Δ PS, respectively) have been generated according to the general procedure outlined above. This was achieved by a series of clonings into bacterial plasmids, followed by recombination of suitable fragments of the viral genome performed in *E. coli*, to generate bacterial plasmids harboring protease deleted adenovirus genomes. This procedure is summarized in Fig. 2 and Fig. 3 and discussed in more detail in the following Examples.

Ad5- Δ PS mutant is deleted for the protease gene only. Ad5CMVLacZ-CMVGFP- Δ PS is deleted for the protease gene, but also in E1 and E3 coding regions of the Ad5 genome. Both mutants have been successfully propagated in the novel complementing cell lines of the invention expressing the Ad2 protease. Ad5CMVLacZ-CMVGFP- Δ PS mutant contains in its genome two exogenous genes (transgenes): the gene of *E. coli* β galactosidase (β gal) and the gene of *Aequorea victoria* green fluorescent protein (GFP). These reporter genes can be easily replaced with genes of therapeutic interest by methods known to those skilled in the art. In both mutants genes of therapeutic interest can be easily introduced by recombination, as both were cloned in bacterial plasmids.

One of the most widely used method to generate recombinant adenovirus vectors involves the transfection of restriction enzyme digested, deproteinized (naked) DNA comprising most viral genes and the right viral DNA terminus, in combination with a transfer vector containing the desired expression cassette, the left viral DNA terminus, and a segment of Ad sequences common to both molecules which permits homologous recombination (Stow, N.D., *J. Virol.*, 37, 171 - 180 (1981)). In typical experiments, co-transfection of 1µg of naked viral DNA with the same amount of transfer vector using calcium phosphate precipitation generates approximately 10 - 30 recombinant plaques with an efficiency of 20 to 60% of recombinants (Jani et al., *J. of Virological Methods*, 64, 111 - 124 (1997)). Such a low efficiency is not sufficient for generation of a library of recombinant adenoviral vectors.

The present invention overcomes this limitation, by allowing, in a preferred embodiment, an easy and efficient generation of E1-deleted, protease-deleted recombinant adenoviral vectors, comprising an exogenous gene or other expressible piece of exogenous DNA in E1 coding region. This is achieved by providing the protease gene (together with exogenous gene or expressible DNA) as part of a dicistronic or independent cassette in place of E1 coding region in a shuttle vector. *In vivo* recombination of the shuttle vector with a protease-deleted adenoviral genome generates viable recombinants only when rescuing the protease gene cloned in E1 coding region. Non-recombinant adenoviral genomes are unable to grow due to protease deletion, which results in elimination of the parental protease-deleted adenovirus after one round of replication. This positive selection ensures an easy generation of a large number of high purity recombinant adenovirus vectors.

It would be obvious to those skilled in the art that other essential late genes, not only the protease gene, could be used for the practice of the invention and that such an essential gene could be expressibly cloned into any transcriptional region of the adenoviral genome, in particular into any of the early transcriptional regions (E1 – E4). It is also not necessary that the exogenous gene be expressed from the same expression cassette (a dicistronic expression cassette) as the essential gene. The exogenous gene

may be expressed from its own expression cassette, under control of either the same or a different promoter as the essential gene is. In either expression cassette (or both), the promoter may be a regulatable promoter, particularly an inducible promoter, such as a tetracycline-inducible promoter.

5

The recombinant adenovirus vectors (AdVs) according to the invention, when comprising a therapeutic exogenous gene, are particularly useful for gene therapy and vaccination. However, the recombinant adenovirus vectors of the invention may be used to express any arbitrary fragments of expressible DNA, such as DNA fragments
10 resulting in expression of antisense RNA fragments of a protein gene. Other expressible DNA fragments could be cis-acting elements regulating gene expression, such as promoters (TATA boxes), enhancers, suppressors, IRES, polyA, termination sequences, UTR sequences of messages that regulate the stability and/or transport of the mRNA (reviewed in Mullick and Massie, *In* :The Encyclopedia of Cell Technology,
15 Editor in Chief, Raymond E. Speir, Wiley Biotechnology Encyclopedias (2000) pp 1140-1164). Such recombinant vectors are particularly useful for the generation of various adenoviral expression libraries.

The use of the PS gene as a positive selection factor is an easy, fast, and cost-efficient
20 means to generate recombinant AdV. Up to now, screening problems have hampered the construction of AdV-based libraries, because of the difficulty to generate high numbers of pure viruses. With the system of the present invention, a high diversity of genes or other expressible DNA fragments can be rapidly expressed by AdVs. Since infection with protease-deleted Ad and transfection of the transfer vectors are readily
25 scalable to more than 10^9 cells (Durocher et al., in: Recombinant Protein Production with Prokaryotic and Eukaryotic Cells. A Comparative View on Host Physiology, Kluwer Academic Publishers, Dordrecht, in press (2001)), the method of the present invention can allow for the construction of libraries with diversity exceeding 10^6 clones.

30

EXPERIMENTAL

The cell lines and vectors of the present invention have been prepared using techniques well known to those skilled in the art. The following examples are provided for better
5 illustration of the invention.

MATERIALS AND METHODS

Cells and viruses

10 293 cells are human embryonic kidney cells expressing high levels of the adenovirus 5 E1A and E1B products (Graham et al., *J. Gen. Virol.*, 36, 59-72 (1977)). 293S cells, a non-adherent 293 cells clone has been previously described (Garnier et al., *Cytotechnology*, 15, 145-155 (1994); Massie et al., *Bio/Technology*, 13, 602-608 (1995)). 293-tTA cell line was described by Massie et al. (*J. Virol.*, 72, 2289-2296
15 (1998)), and the 293-rtTA cell line was obtained in a similar way. Adenovirus Ad2ts1 mutant was previously described (Weber, *J. Virol.*, 17, 462-471 (1976)). Adenovirus dl309 is a fully replicative mutant and was previously described (Jones et. al., *Cell*, 13, 181-188 (1978)). AdCMV5-GFP is a recombinant adenovirus in which E1 region has been replaced by a CMV driven GFP expression cassette (Massie et al.,
20 *Cytotechnology*, 28, 53 - 64 (1998)).

Plasmids

Plasmid pTKNeo was generated by auto-ligation of the BstEII fragment of pREP 9 (Invitrogen). Plasmid pTR-DC/GFP was previously described (Mosser et al, 1997). This
25 plasmid has been modified from pUHD10.3 (Resnitsky et al., *Mol. Cell. Biol.*, 14, 1669-1679 (1994)) which contains the tTA-responsive promoter with a dicistronic expression cassette. Dicistronic expression is permitted by the encephalomyocarditis virus IRES (Ghattas et al., *Mol. Cell. Biol.*, 11, 5848-5859 (1991)). The original pTR-DC/GFP was modified by insertion of a BglII site. Protease gene was excised from pAdBM5-PS, by
30 BamHI digestion, sequenced and subcloned into the BglII site of pTR-DC/GFP. Final plasmid, pTR5/PS-DC/GFP thus co-expresses inducibly GFP S65T mutant and Ad2

protease genes. Expression of GFP and protease were assayed by transfection in 293 cells. The transient expression of the protease was established by Western-blot with an anti-protease polyclonal antiserum raised in rabbit with a recombinant protein (from Dr J. Weber, University of Sherbrooke). The expressed protein had the same molecular weight as the native protein from wild-type adenovirus, and was expressed only when induced. Plasmid pDE3 was a gift of Dr Lochmüller (Montreal Neurological Institute). This plasmid contains the right end of Ad5 genome from the BamHI site (21562) to the end of the genome, with an E3 deletion. This deletion corresponds to the one described by Bett et al. (1994) and originates from plasmid pBHG11 (extent of the deletion: 27865-30995). Plasmid pAdEasy-1- β Gal-GFP was a gift of Dr He (John Hopkins University, Baltimore, MD) and has been already described (He et al., 1998). Plasmid pTG3602 (Chartier et al., 1996) was a gift of Dr Mehtali (Transgene SA, Strasbourg, France). Recombinant adenovirus construction in *E. coli* was performed as described respectively by He et al. (1998) and Chartier et al. (1996).

Generation of protease expressing cell lines

293-tTA cell lines were generated by co-transfection of pTR5/PS-DC/GFP and pTKNeo. 293 rTA-PS clones were generated in a similar way by co-transfecting the same plasmid with the p3'SS (Stratagene) in 293S rTA. Transfections were achieved by the optimized calcium phosphate precipitation method (Jordan et al., *Nucleic Acids Res.*, 15, 24(4): 596-601(1996)). For tTA and rTA, selection drugs were respectively G418 and hygromycin (Sigma Chemical).

Selection of recombinant cell clones

After co-transfection and selection, clones of 293S cells expressing the GFP from the dicistronic cassette were selected by screening for the expression of the GFP by flow cytometry analysis and cell sorting. Flow cytometry was performed using an EPICS Profile II (Coulter, Hialeah, FL, USA) with a 15 mW argon-ion laser. Cell sorting was carried out on an EPICS V (Model 752, Coulter) multiparameter laser flow cytometer and cell sorter, using the Auto-clone (multiwell automated cell deposition) system. Before selection and sorting, expression of both GFP and protease was induced by

addition (rtTA) or suppression (tTA) of doxycycline. For the analysis of GFP expression, cells were sterily collected and concentrated (1×10^6 cells/ml) in phosphate-buffered saline (PBS) by centrifugation. The mostly fluorescent cells were gated and distributed clonally in 96-well plates.

5

Analysis of recombinant protein expression

Expression of the GFP was checked periodically by flow cytometry analysis, while expression of the protease was assayed by western-blotting. Cells were washed in PBS, centrifuged and frozen. Lysis was carried out in 100 mM Tris-HCl [pH 6.9], 10% glycerol, 2% SDS, and high molecular weight DNA was disrupted by sonication. Prior to assay, total protein contents of extracts were titrated using the DC Protein Assay Kit (Biorad). For electrophoresis, samples were diluted in Laemmli buffer (Laemmli et al., *J. Mol. Biol.*, 88, 749-165, (1974)) and boiled for 5 min. An estimated 20 μ g total protein quantity was loaded per well in 14% acrylamide:bisacrylamide (30:1) gels. After electrophoresis, proteins were transferred to nitrocellulose membranes which were subsequently blocked overnight at 4°C with PBS containing 5 % nonfat dry milk, 0.1 % Tween 20. The rabbit anti-protease antibody was diluted 1:20000 in the same buffer but with 0.2% Tween 20. As an internal control, an anti-actin monoclonal antibody diluted 1:10000 was used. Incubation was overnight at 4°C. Conjugates were used at a 1:10000 dilution in the same buffer for 1 hr at room temperature. Revelation was carried out using the ECL chemiluminescence kit (Amersham) according to the manufacturer's instructions.

15

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EXAMPLES

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Generation and isolation of 293 cell lines transformed with Ad2 protease gene

Cell lines were generated by co-transfection and selection with appropriate agents as summarized in Table 1.

30 Table 1

Analysis of the clones obtained from transformation of 293 cells with protease

Cells	Plasmids used for transfection	Selection agent	Clones obtained	Clones analyzed	Selected Positive clones
293 tTA	pTR5/PS-DC/GFP pTKNeo	+ G418	>50	17	7
293 rTA	pTR5/PS-DC/GFP p3'SS	+ hygromycin	>50	14	9

293 tTA cells were co-transfected with pTR5/PS-DC/GFP and pTKNeo, while 293 rTA were co-transfected with the same plasmid and p3'SS. After a 48 hour recovery, transfected cells were submitted to a three weeks selection by either G418 (500 µg/ml) for 293 tTA or hygromycin (150 µg/ml) for 293 rTA. During this time, fresh medium and drug were applied to cells twice a week. Throughout the selection process, GFP expression was monitored on aliquots by flow cytometry analysis. Cells were then sorted using the multiwell automated cell deposition system and clonal distribution was visually checked. Expression was then assessed and only homogenous clones (as checked by unicity of the peak of fluorescent cells) were selected. Results of GFP expression of stable clones are summarized in Table 2.

Table 2

15 GFP expression and induction efficiency in 293-tTA-PS and 293-rTA-PS

Clone	FI		Induction Factor
	OFF	ON	
293-tTA-PS-2	138	2838	20
293-tTA-PS-11	63	1322	21
293-tTA-PS-15	30	3186	106
293-rTA-PS-7	35	1228	35
293-rTA-PS-10	107	720	7
293-rTA-PS-17	118	1654	14

Selected cell line clones were tested for the expression of the GFP (basal and induced) by flow cytometry analysis. FI: fluorescence index calculated as the percentage of cells expressing GFP by the mean fluorescence value; OFF: GFP expression without induction (50 ng doxycycline per ml for tTA); ON: GFP expression after induction (1 µg per ml for rtTA). Induction factor was calculated as the ratio between the FI of the ON state and the FI of the OFF state.

Of all the tested clones, three of 293S-tTA-PS and three of 293S-rtTA-PS clones were selected. Induction efficiency was measured by comparing products of the mean fluorescence of one cell by the percentage of fluorescent cells (fluorescence indexes: FI). Induction factors ranged from 7 to 106 which is in the range of what is usually observed with tetracycline-regulated expression cassettes.

Protease expression in 293-tTA-PS and 293-rtTA-PS

Of the clones tested for protease expression, three clones of 293-tTA-PS and of 293-rtTA-PS are presented (Fig. 1A). Expression was revealed with a polyclonal rabbit anti-serum raised to the *E. coli*-expressed protein. Results demonstrate that the expressed protein displays the same electrophoretic pattern than that of the endogenous adenovirus protein, and that the expression depends on the induction in all tested clones. The latter assertion was checked by the internal control (cellular actin) which demonstrates that the same amount of protein extract has been loaded in each well. Testing of all obtained clones of rtTA did not allow for observation of higher levels of expression than that reached with tTA clones. Level of expression of protease in all selected clones were equal or higher than that of the native adenovirus protease (Fig. 1A, lane 3). To check that, 293 cells were infected at a MOI of 10 pfu with AdCMV5-GFP (Massie et al., *Cytotechnology*, 28, 53 – 64 (1998)) and protein extracts were prepared at 48 hrs p.i. Similar level of expression was achieved with the clones derived from 293-tTA and 293-rtTA cells.

Biological activity of protease expressed by cell lines

To study the biological activity of Ad2 protease in transformed cell lines, complementation of the temperature-sensitive Ad2ts1 and of two novel protease deleted mutants by the cell lines was examined. Ad2ts1 viral particles produced at 39°C contain a functionally deficient protease, and they were used to assess complementation. Results of one-step growth curves in 293 and 293-PS cell lines (tTA and rtTA) for Ad2ts1 are summarized in Table 3 and for both novel protease deleted mutants in Table 4.

Table 3

Yield of dl309 and Ad2ts1 from One-Step growth curves in different 293-derived cell lines

Virus	Cell line	Temperature	Virus titer
dl309	293-tTA	33	1.7×10^8
		39	1.2×10^8
	293-tTA-PS-15 NI	33	2.0×10^8
		39	1.2×10^8
	293-tTA-PS-15 I	33	8.1×10^7
		39	7.0×10^7
	293-rtTA	33	1.6×10^8
		39	1.5×10^8
	293-rtTA-PS-7 NI	33	1.5×10^8
		39	1.0×10^8
	293-rtTA-PS-7 I	33	7.8×10^7
		39	7.2×10^7
Ad2ts1	293-tTA	33	2.5×10^8
		39	5.0×10^3
	293-tTA-PS-15 NI	33	1.6×10^8

	39	9.0×10^7
293-tTA-PS-15 I	33	4.0×10^7
	39	5.0×10^7
293-rTA	33	2.0×10^8
	39	2.0×10^3
293-rTA-PS-7 NI	33	1.5×10^8
	39	3.2×10^8
293-rTA-PS-7 I	33	8.0×10^7
	39	5.8×10^7

Cells were infected at a multiplicity of 2 plaque-forming unit (p.f.u) per cell. 2-3 days later at 39°C or 5 days later at 32°C, cells were harvested, frozen-thawed three times, and subsequent extracts were titrated. Results of a typical experiment are presented here. Titers were determined as p.f.u. on 293 cells at 33°C. NI: non-induced, I: induced expressions. Experiments carried out at 33°C were included as controls.

While Ad2ts1 yielded respectively 5×10^3 p.f.u. and 2×10^3 p.f.u. at 39°C in 293-tTA and 293-rTA cell lines, complementation was evidenced by the obtention of titers similar to that of the dl309 mutant in protease expressing cell lines. Induction had the effect of slightly decreasing titers, but surprisingly, basal expression of the gene from 293-tTA-PS-15 and 293-rTA-PS-7 was sufficient to complement the Ad2ts1 mutant. There was no difference between tTA and rTA complementing cell lines.

To further demonstrate the biological activity of cell lines and to characterize novel Ad5 mutants, one-step growth curves in 293-tTA/rTA and 293-tTA/rTA-PS cell lines were generated (Table 4).

Table 4

Yield of Ad5CMVLacZ-CMVGFP-ΔPS and of Ad5-ΔPS with corresponding controls from One-Step growth curves in different 293-derived cell lines.

Virus	Cell line	Virus Titer
Ad5CMVLacZ-CMVGFP-ΔPS	293-tTA/rTA	$<10^4$
	293-rTA-PS-7 NI	10^8
	293-rTA-PS-7 I	$6.0 \cdot 10^7$
	293-tTA-PS-15 NI	$1.4 \cdot 10^8$
	293-tTA-PS-15 I	$5.0 \cdot 10^7$
Ad5CMVLacZ-CMVGFP	293-tTA/rTA	$6.3 \cdot 10^8$
	293-rTA-PS-7 NI	$6.5 \cdot 10^8$
	293-rTA-PS-7 I	$1.2 \cdot 10^8$
	293-tTA-PS-15 NI	$5.0 \cdot 10^8$
	293-tTA-PS-15 I	10^8
Ad5-ΔPS	293-tTA/rTA	$<10^4$
	293-rTA-PS-7 NI	$1.4 \cdot 10^8$
	293-rTA-PS-7 I	$7.0 \cdot 10^7$
	293-tTA-PS-15 NI	$1.5 \cdot 10^8$
	293-tTA-PS-15 I	$5.2 \cdot 10^7$
Ad5	293-tTA/rTA	$6.0 \cdot 10^8$
	293-rTA-PS-7 NI	$6.5 \cdot 10^8$
	293-rTA-PS-7 I	$1.0 \cdot 10^8$
	293-tTA-PS-15 NI	$6.5 \cdot 10^8$
	293-tTA-PS-15 I	$1.0 \cdot 10^8$

Cells were infected at a multiplicity of 2 plaque-forming unit (p.f.u) per cell. 2-3 days later, cells were harvested, washed three times in PBS, frozen-thawed three times, and subsequent extracts were titrated. Results of a typical experiment are presented here.

5 Titers were determined as p.f.u. on 293 cells. NI: non-induced, I: induced expression.

Biological activity was also demonstrated by the ability of the 293-rTA-PS-7 clone to generate protease deleted mutants Ad5CMVLacZ-CMVGFP-ΔPS and Ad5-ΔPS after transfection of recombinant DNA. As expected, while the protease deleted mutants
10 were unable to grow in 293-rTA, complementation by cell lines allowed for the restoration of viral titers close to those of the controls (which are exactly the same viruses as the mutants, except for the presence of the protease gene). It is noteworthy that as for Ad2ts1, basal protease expression from both tTA and rTA complementing cell lines is sufficient to complement the protease deleted mutants. It is also noteworthy

that in the induced state of expression of the protease, viral yields were slightly decreased.

Growth rate of 293-PS cell lines

- 5 Visual examination of 293-PS cell lines showed that after induction of expression cells did not displayed a significantly different phenotype. To further study the effect of induction on cell lines, viability of cells was measured by counting living cells, either induced or not, after trypan blue staining every day from D0 to D5. For 293-rtTA-PS cells, there was no difference between induced or non-induced cells. For one clone of
- 10 293-tTA-PS (clone 2), results are represented in Figure 4. It can be seen that the expression of the protease had no significant deleterious effect on cells growth. It is clear as well that as no effect could be evidenced during a period (24-48 hrs) compatible with the production of a recombinant adenovirus mutant, these cell lines will be useful for generation and expansion of protease-deprived mutants. Expression of the
- 15 gene did not show a toxic effect, but rather a slight cell growth impairment: when maintained in the induced state of protease expression, cells grew slower. Given that overexpression of the protease could slightly impair cell growth as well as reducing viral yields, controlling its expression with a regulatable promoter was paramount both for obtaining the best protease complementing cell lines as well as for insuring maximal
- 20 production of protease deleted AdV.

Stability of 293-PS cell lines

- To check the stability of selected clones, cell lines maintained during 2 months without selection drug were assayed for the expression of the GFP and of the protease. Both
- 25 proteins were expressed at levels similar to that of early passage cells as determined by respectively flow cytometry and immunoblot analyses (data not shown). No change in drug susceptibility was noticed after 2 months passages and neither did protease expression levels were modified.

Transfectability of 293 PS cell lines

Clones 293-tTA-PS-15 and 293-rtTA-PS-7 were analyzed for the ability to support the production of viral plaques after transfection with AdCMV-LacZ DNA. Both clones yielded as many viral plaques as respectively parental 293-tTA and 293-rtTA cells. 293PS cell lines were thus very efficiently transfected and were subsequently used for the generation of protease-deleted mutants.

Effect of adenovirus infection on protein expression

To study the effect of the expression of IVa2 products (Lutz et al., *J. Virol.*, 70, 1396-1405, (1996)) on the MLP enhancer that is included in our construction, cell lines were infected in triplicate at a MOI of 1 p.f.u. and GFP expression was followed in induced and non-induced cells. No significant difference could be evidenced between both batches.

Generation of protease-deleted mutants

Plasmids clonings are summarized in Fig. 2. For the construction of protease-deleted mutants of Ad5, an extension sufficient for homologous recombination was first introduced in pDE3 plasmid by ligation of the 6145 bp fragment resulting from the RsrII/XhoI digestion of the Ad5 genome into the unique sites Sall and XhoI. To clone this insert and generate pDE3-ext plasmid, RsrII (from the insert) and Sall (from pDE3) were first T4 DNA polymerase repaired. Protease deletion was engineered by PCR to synthesize a 171 bp upstream fragment (forward primer: gtcgacCATGGACGAGCCCACCCTTCT, SEQ. ID NO: 1 reverse primer: ggatccGGCGGCAGCTGTTGTTGATGT) SEQ. ID NO: 2 and a 2448 downstream fragment (forward primer: agatctAAATAATGTACTAGAGACACT, SEQ. ID NO: 3 reverse primer: ctcgagTTCCACCAACACTCCAGAGTG) SEQ. ID NO: 4 (Restriction sites added for cloning purposes are shown in lower case.) These fragments were cloned in the pDE3 plasmid in the Sall and XhoI sites of the plasmid, using the BamHI/BglII ligation compatibility. The SfiI/BamHI fragment from this plasmid was subcloned into pSL1190 plasmid (Pharmacia) and sequenced. It was subsequently

cloned into pDE3-ext plasmid in the same sites, generating pDE3-ext-ΔPS plasmid. To generate protease-deleted Ad mutants, recombination into *E. coli* was chosen (Fig. 3). An E1/E3 deleted mutant: Ad5CMVLacZ-CMVGFP-ΔPS was constructed in plasmid by cotransfection in *E. coli* of the NdeI/XhoI fragment from pDE3-ext-ΔPS with pAdEasy1-βgal-GFP SgfI digested. A mutant deleted only for the protease (Ad5-ΔPS) was generated in the same manner from pTG3602 plasmid. Seven micrograms of plasmid DNA PacI digested from both pAdEasy1-βgal-GFP and pTG3602-ΔPS were transfected in 293-PS-rtTA-7 cell line clone to generate recombinant protease-deleted mutants. The same amount of recombinant linearized plasmid DNAs were also transfected in 293 and 293-rtTA cells as controls. As expected, this experiment yielded no viral plaques. After 10-14 days viral plaques were observed in 293-PS-rtTA. As recombinant adenoviruses have been generated in *E.coli*, no further cloning of plaques was required (He et al, 1998; Chartier et al, 1996). The whole monolayer was scraped and virus was released from cells by freeze-thaw cycles. All viral plaques of recombinant virus displayed no phenotypic differences from that of wild-type virus.

Ability of Ad5-ΔPS mutant not deleted for E1 to perform a single round of replication in non-complementing cell lines.

To demonstrate the ability of the mutant deleted for the protease and not for E1 (Ad5-ΔPS) to perform a single round of replication in non-complementing cell lines, A549 cells were inoculated with wild-type, Ad5-ΔPS, AdΔE1.E3, and Ad5CMVLacZ-CMVGFP-ΔPS viruses. Comparison of viral protein production (Fig. 6) and of viral yields (Fig. 7) of the different viruses show that only the mutant deleted for the protease and not for E1 is able of undergoing a single round of replication in non-complementing cells.

Selection of recombinant AdV by ectopic expression of the PS gene

Adenovirus has terminal proteins covalently linked to its ITRs, which enhances its infectivity by more than 100-fold above what is obtained with naked DNA. As a first step towards the construction of AdV libraries, the efficacy with which recombinant AdV could be obtained following co-transfection of a transfer vector with viral DNA-TPC was evaluated. Combining published protocols (Miyake et al., *Proc. Natl. Acad. Sci. USA*, 93,

1320 - 1324 (1996); Okada et al., *Nucl. Acids Res.*, 26, 1947 – 1950 (1998)), viral DNA-TPC was purified. The best of several preparations yielded approximately 150000 plaques/μg of uncut Ad DNA-TPC. Generation of recombinant AdV was compared for Ad DNA-TPC and naked Ad DNA. After digestion with *ClaI* and co-transfection with a transfer plasmid expressing GFP (pAdCMV5-GFPq), at least 100 times more recombinants were generated with DNA-TPC (that is 3500-5000 plaques/μg) than with naked viral DNA. The ratio of recombinant/non-recombinant plaques was about 60% in two separate experiments.

10 As PS-deleted Ads are capable of only one round of replication in 293 cells (Oualikene et al., *Hum. Gene Ther.*, 9, 1341 - 1353 (2000)), this characteristic was exploited to develop a positive selection method, i.e., whether recombinant vectors could be efficiently selected using ectopic expression of the PS gene in the E1 region following recombination (Fig. 5). As it had been shown previously that minute amounts of PS could fully complement PS-

15 deleted Ad while over-expression of PS could be deleterious to cells (Oualikene et al., *supra*), two promoters of different strength were tested. In both cases, the PS gene cloned in an Ad transfer vector was expressed from a tetracycline-inducible promoter containing either the TATA box of the CMV promoter (TR5) (Massie et al., *J. Virol.*, 72, 2289 - 2296 (1998)) or the weaker TATA box of the TK promoter (TR6), in a dicistronic cassette co-

20 expressing GFP (Massie et al., *Cytotechnology*, 28, 53 - 64 (1998)). Co-transfection of linearized pAdTR5-PS-GFPq and pAdTR6-PS-GFPq transfer vectors with *ClaI*-digested DNA-TPC of Ad5-ΔPS in 293 cells allowed for generation of viral plaques that appeared as early as 5 days after co-transfection. One hundred viral plaques for each transfections were checked by microscopic examination for GFP expression directly on the transfected

25 plates or after infection of 293-rTA cells to increase the GFP signal by induction of the promoter. All generated plaques were GFP positive after one round of multiplication on 293-rTA under induced conditions. Two independent clones originating from the transfection with pAdTR5-PS-GFPq and pAdTR6-PS-GFPq respectively, were randomly chosen and plaque-purified once to further assess their purity. In both cases, 100% of the

30 resulting plaques (175/175) were GFP positive. Therefore, ectopic expression of the PS

gene with either promoter worked equally well for the positive selection of recombinant AdV, even under uninduced conditions.

Determination of PS expression effect on viral progeny yields

5 Since the PS gene is expressed in recombinant AdV from a different promoter, the growth of AdV ectopically expressing the PS compared to the wild-type Ad5 was tested, in order to ensure that the positive selection method was not affecting the growth of the resulting AdV. Under uninduced conditions, for both promoters (TR5 and TR6) the AdV progeny grew as efficiently as a normal virus. However, for AdTR5-PS-GFPq the yield was reduced
10 by about 6-fold following induction at 5 hours, while induction at 24 hours had no effect on viral growth (Fig. 8). The 2-fold reduction in viral progeny observed with the E1-deleted AdV as compared to wild-type Ad5, is due the expression of the E1 region by the parental virus which is not fully complemented for E1-deleted AdV in 293 cells (unpublished results).

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The reduction of viral progeny from AdTR5-PS-GFPq-infected cells under induced conditions at early time was expected, because it had been previously demonstrated that premature activation of PS by addition of pVlc can significantly reduce viral titers (Rancourt et al., *Virology*, 209, 167 - 173 (1995)). This was simulated in this experiment by
20 the inappropriate timing of PS over-expression by induction 5 hours after infection. Furthermore, as shown in Fig. 9, the level of PS expression from AdTR5-PS-GFP following induction is much higher than its level without induction, which turned out to be similar to the one seen in Ad5 infection (Fig. 2, lane 8). By contrast, the expression level of PS from AdTR6-PS-GFP following induction is similar to the level seen in Ad5 infection,
25 and about 10-fold lower in uninduced conditions. These data confirm previous results which showed that lower level of expression of PS was enough to complement Ad5- Δ PS for replication, while premature over-expression of PS was deleterious to viral growth (Oualikene et al., *supra*). Interestingly, in the case of AdTR6-PS-GFPq, no significant difference was found after early induction, yet the growth of the resulting AdV was normal.
30 The fact that a much weaker promoter can be used will ensure that in functional studies of transgenes in non permissive cells, the very low levels of expression of the PS gene will

not interfere with the cell physiology in any significant ways. In fact, this expression level will be much lower than what is well tolerated in 293-PS cells lines under uninduced conditions (Fig. 9, lane 10), since the amount of PS produced in AdTR6-PS-GFPq-infected 293 cells is expressed from 10^5 copies of the gene subsequent to viral DNA replication, whereas it will be 100-1000 times lower in non-complementing cells at low MOI (Massie et al., *supra*).

Construction of recombinant AdVs by the Infection/Transfection protocol

Although efficient at generating large number of recombinant plaques with the PS selection, the production of significant quantities of pure digested viral DNA-TPC is expensive and time-consuming. This could represent an obstacle for the generation of larger libraries requiring significant amounts of viral DNA-TPC for the transfection of much higher number of cells. Given the efficiency of the PS selection, it was decided to simplify the method by directly delivering the Ad5- Δ PS genome by infection in combination with transfection of the transfer vector expressing the PS gene. Since GFP+ plaques could be more readily detected with pAdTR5-PS-GFPq in 293 cells (uninduced conditions), this transfer vector was used to establish the optimal conditions for this infection/transfection protocol.

In a first series of experiments, 293 cells were either first infected with Ad5/ Δ PS and transfected with pAdTR5-PS-GFPq 5 hours later, or first transfected and infected after overnight (O/N) incubation. The infection was carried out at different MOI ranging from 10^{-2} to 10^{-7} and cells harvested at 5 days post-infection or post-transfection, as the case may be. The results from the Inf/Trans and Trans/Inf methods were compared by titrating viral yields by plaque assays (Fig. 10). The correlation between the resulting AdV titer and MOI used was sigmoidal with a peak at a MOI of 10^{-3} for both conditions. In this particular experiment, a significant decrease was observed at MOI of 10^{-2} . However, in other separate experiments, this decrease was not seen at the MOI of 10^{-2} but rather at 10^{-1} . In any case, this indicated that there was an upper limit to the amount of Ad5/ Δ PS that can be used to obtain the best titer of recombinant AdV. Overall, both methods being equivalent, the Inf/Trans method was further investigated.

In another series of experiments, 293 cells were infected with Ad5-ΔPS at MOIs of 10^{-2} , 10^{-3} and 10^{-4} , and harvested at different times ranging from 1 to 6 days post-transfection with pAdTR5-PS-GFPq. The results shown in Fig. 11 indicated that the recombinant AdV are first detected at day 2 and their number steadily increased up to day 6. The best titer at day 4 was obtained with a MOI of 10^{-2} . However, the difference in yields at day 6 is less accentuated for the different MOIs, while at that point the best titer was obtained with a MOI of 10^{-3} . This indicated that the optimal MOI could varied within one order of magnitude without significant fluctuation in the yield. Unfortunately, it was not possible to establish the number of recombination events since as soon as a recombinant AdV is generated it will produce a progeny which will increase the total number of recombinants. Thus, the increase in total number of recombinants from days 2 to 6 could be due to both new recombination events as well as amplification of recombinants generated the previous days.

Determination of library diversity

In order to determine the potential diversity of AdV libraries generated by the Inf/Trans method, an experiment in 96 well plates was carried out to establish the minimal number of cells required for one recombination event. A series of two-fold dilution of cells from 10^4 per wells were infected with 4 different MOIs from 10^{-2} to 1.25×10^{-3} and the cells harvested 5 days post-transfection with pAdTR5-PS-GFPq. Fifty percent of the lysate from each well was then used to infect 293 cells in 96 well plates and the presence of recombinant AdV was assessed by scoring GFP+ cells. The results are presented in Fig. 12. At 5000 cells/well the variation of yield at various MOI was minimal, all wells (10/10) being GFP+ except at the lowest MOI. At the optimal MOI of 10^{-2} , one recombination event could be detected with as little as 625 cells in 6 out of 10 wells. In accordance with the Poisson distribution, this data can be interpreted as an indication that one recombination event had occurred in less than 10^{-3} cells.

In a further experiment, the representativeness of libraries generated either by co-transfection of viral DNA-TPC with a mixture of transfer vectors or by the Inf/Trans

method with positive selection with the PS gene was compared. To simulate a real library, the mixture of transfer vectors was effected by mixing different ratio of bacteria harbouring the various plasmids and extracting the DNA from the pool of the mixed populations. As shown in Table 5, thousand of plaques were generated by co-transfection with viral DNA-TPC, of which about 80% were recombinants. It is noteworthy that the ratio of BFP/GFP plaques was fairly representative of the initial input of bacterial clones harboring the respective transfer vectors with a diversity approaching 1 in 500 in this particular experiment. With the Inf/Trans method, 100% of the plaques were recombinants while, as it can be seen in Table 6, that the ratio of GFP-/GFP+ clones was also consistent with the ratio of input transfer vector DNA.

Table 5

Analysis of the representativeness of a mini AdV library generated by co-transfection with viral DNA-protein complex.

BFP+/GFP+ (ratio of DNA)	Total number of plaques	Percentage of recombination	BFP+ plaques	GFP+ plaques	BFP+/GFP+ (ratio of plaques)
100/1	1000	75%	736	14	52.6/1
500/1	1100	84%	920	4	230/1

The 293 cells were co-transfected with AdΔE1ΔE3/ DNA-TPC *ClaI* digested and two different ratio of the pAdCMV5BFPq/pACMV5GFPq *ClaI* digested. The following day, the transfected cells were split 1/10 with fresh cells, and overlaid with agarose. The number of total plaques, percentage of recombination, and the ratio of BFP+/GFP+ plaques are presented in this table.

Table 6

Analysis of the representativeness of a mini AdV library generated by the Inf/Trans method with positive selection by ectopic expression of the PS gene

GFP-/GFP+ (ratio of DNA)	GFP- plaques	GFP+ plaques	GFP-/GFP+ (ratio of plaques)
50/1	2500	72	34.7/1
100/1	1838	27	83.5/1

- 5 293 cells were infected with Ad5-ΔPS at a MOI of 10^{-2} and then transfected with two different ratio of the pAdTR5-PS/pAdTR5-PS-GFPq *FseI* digested. The cells were harvested 3 days post-infection, freeze-thawed and seeded on 293 cells at the appropriate dilution to yield well isolated plaques (around 10^{-2}). The number of GFP+ and GFP- plaques and their ratio are presented in this table.

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The following cell line was deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209 on December 3, 1998 and assigned the following accession number:

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complementing cell line 293 rTA.PS.7 ATCC CRL-12595

The following adenovirus mutants were deposited with the American Type Culture Collection on May 5, 1999 and assigned the following accession numbers:

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human adenovirus type 5 mutant Ad5-ΔPS ATCC-VR-2640
human adenovirus type 5 mutant Ad5CMVlacZ-CMVGFP-ΔPS ATCC-VR-2641

25 The following plasmids and recombinant adenovirus mutants were deposited with the International Depository Authority of Canada, Bureau of Microbiology, Health Canada,

1015 Arlington Street Winnipeg, Manitoba, Canada R3E 3R2 on Feb, 1 2000 and assigned the following accession numbers:

	transfer plasmid pAdTR5-PS-GFPq	IDAC010201-1
5	transfer plasmid pAdTR6-PS-GFPq	IDAC010201-2
	recombinant adenovirus mutant rAdTR5-PS-GFPq	IDAC010201-3
	recombinant adenovirus mutant rAdTR6-PS-GFPq	IDAC010201-4

10 Although various particular embodiments of the present invention have been described herein before, for purposes of illustration, it would be apparent to those skilled in the art that numerous variations may be made thereto without departing from the spirit and scope of the invention, as defined in the appended claims.